Kinetics of Sugar Transport and Phosphorylation Influence Glucose and Fructose Cometabolism by *Zymomonas mobilis*†

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The competitive inhibition of fructokinase by glucose has been proposed as the mechanism by which *Zymomonas mobilis* preferentially consumes glucose from mixtures of glucose and fructose and accumulates fructose when growing on sucrose. In this study, incorporation of radioactive fructose into biomass was used as a measure of fructose catabolism. It was determined that the rate of fructose incorporation by *Z. mobilis* CP4 was somewhat lower in the presence of an equimolar concentration of glucose but that the inhibition of fructokinase by glucose was not nearly as severe in vivo as was predicted from in vitro studies. Interestingly, addition of glucose to a culture of *Z. mobilis* CP4-M2, a glucokinaseless mutant, resulted in an immediate and nearly complete inhibition of fructose incorporation. Furthermore, addition of nonmetabolizable glucose analogs had a similar effect on fructose catabolism by the wild-type *Z. mobilis* CP4, and fructose uptake by *Z. mobilis* CP4-M2 was shown to be severely inhibited by equimolar amounts of glucose. These results suggest that competition for fructose transport plays an important role in preferential catabolism of glucose from sugar mixtures. Indeed, the apparent K_m values for sugar uptake by *Z. mobilis* CP4 were approximately 200 mM for fructose and 13 mM for glucose. Other experiments supported the conclusion that a single facilitated diffusion transport system, encoded by the *glf* gene, is solely responsible for the uptake of both glucose and fructose. The results are discussed with regard to the hypothesis that the kinetics of sugar transport and phosphorylation allow the preferential consumption of glucose and accumulation of fructose, making the fructose available for the enzyme glucose-fructose oxidoreductase, which forms sorbitol, an important osmoprotectant for *Z. mobilis* when growing in the presence of high sugar concentrations.

Zymomonas mobilis is a gram-negative, obligately fermentative bacterium found in association with plants containing high concentrations of sugars in their saps and fruit juices (16, 27). *Z. mobilis* is unique in employing the Entner-Doudoroff pathway anaerobically; dissimilation of pyruvate to ethanol and carbon dioxide is the sole means for energy generation (4, 11, 16, 27, 29). This pathway yields only a single mole of ATP per mole of sugar fermented, giving *Z. mobilis* the lowest molar growth yield reported for a bacterium (27). In order to cope with this low energy yield, glucose is metabolized at a remarkably high rate (24): each minute *Z. mobilis* consumes an amount of glucose equal to one-third of its mass. Another implicit consequence of this metabolic strategy is that little energy is available for sugar transport systems; glucose is transported by a non-energy dependent, low-affinity, high-velocity facilitated diffusion system (6, 18, 30).

The only sugars that can support the growth of wild-type *Z. mobilis* strains are glucose, fructose, and sucrose, the last of these carbon sources being cleaved outside the cell by various sucrase enzymes to provide free glucose and fructose (19). There are some interesting differences between fructose and glucose metabolism. First, the profiles of fermentation side products for fructose and glucose are very different (29). Second, a significant amount of fructose is converted to the extracellular polymer levan by the enzyme levansucrase when *Z. mobilis* is grown on sucrose (29). Third, *Z. mobilis* has a periplasmic enzyme called glucose-fructose oxidoreductase

(GFOR) (31) which reduces fructose to sorbitol, an osmoprotectant in *Z. mobilis* (12). Fourth, *Z. mobilis* possesses a facilitated diffusion transport system which has approximately 10-fold-higher affinity for glucose than for fructose (6, 18, 30). Fifth, fructokinase and phosphoglucose isomerase activities are elevated by a mechanism involving increased transcription of the corresponding genes (10, 32) when cells are grown on fructose, presumably due to a need to increase the enzyme levels to match the pathway flux (1). Transcription of the glucose facilitator gene, *glf*, is also elevated by growth on fructose, although it is not known if there is a parallel increase in transport activity (2). Lastly, free glucose and fructose are phosphorylated at the expense of ATP by different substrate-specific kinases (7).

When *Z. mobilis* is grown on sucrose, the fructose moiety accumulates (8) and the specific consumption rate of the glucose moiety appears to be 66% higher than that of the fructose moiety (20). Not only does consumption of fructose appear to lag behind that of glucose on sucrose and mixtures of the two sugars, but there is also a corresponding formation of sorbitol from fructose (3, 28). The preferential catabolism of glucose from sucrose or mixtures of glucose and fructose has been attributed to inhibition of fructokinase by glucose; kinetic analysis of purified fructokinase from *Z. mobilis* have indicated that the enzyme is inhibited by glucose with a K_i of 0.14 mM (21). These studies have led to suggestions that fructose is preferentially converted to sorbitol, levan, and other side products rather than being catabolized (together with glucose) for energy and biomass production. Surprisingly, the effect of glucose inhibition of fructokinase on cometabolism of the two sugars has never been tested directly, nor is it known whether genetic regulation of *glf* or differences in the kinetics of glucose and fructose transport contribute to accumulation of fructose. The

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purpose of this study was to more closely examine cometabolism of glucose and fructose mixtures by *Z. mobilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Z. mobilis* strains were grown at 30°C in complex medium (YEP medium) containing various concentrations and mixtures of carbohydrates as described previously (17). Growth was monitored by measuring the absorbance at 550 nm (A_{550}) or A_{600} with a Spectronic 601 spectrophotometer. The glucokinase mutant used in these studies, *Z. mobilis* CP4-M2, was provided by A. Romano (6). A plasmid containing the *Z. mobilis glk* gene, pLOI713, was used for genetic complementation of *Z. mobilis* CP4-M2 (24). Tetracycline was added (10 mg/liter) for maintenance of pLOI713.

Enzyme assay. Fructokinase was assayed by coupling with phosphoglucose isomerase and glucose-6-phosphate dehydrogenase essentially as described previously (1). Cell cultures were harvested, and cell lysates were prepared by passage through a French press, as described previously (17).

Transport and incorporation of radiolabeled sugars. Short term uptake of D-[U- 14 C]glucose and D-[U- 14 C]fructose was measured by using the -3°C quench method as described previously (18). For kinetic measurements, the specific activities of the radioisotopes were varied from 0.4 to 55 $\mu\text{Ci}/\mu\text{mol}$, the concentration of fructose was varied from 1 to 500 mM, and the concentration of glucose was varied from 0.1 to 100 mM. Measurements of radioactive fructose uptake were conducted in the presence of 20 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in order to prevent the uptake of radioactive sorbitol, which can be formed by the enzyme GFOR when glucose and fructose are present (12). For transport inhibition studies, 30 mM radioactive fructose (0.25 $\mu\text{Ci}/\mu\text{mol}$) was mixed with 30 mM cold glucose, and triplicate assays were averaged for each time point. Transport of radioactive xylose (40 mM; 0.25 $\mu\text{Ci}/\mu\text{mol}$) was used as a measure of total sugar uptake activity (xylose is transported by Glf [25]). Multiple time points were used for determination of transport kinetics (initial uptake rates were linear for approximately 20 s), and the data were analyzed by using GraFit software (Erithacus Software, Ltd.) as described previously (18). Incorporation of radioactive fructose into cell components was measured by precipitation with hot trichloroacetic acid (TCA). An overnight culture growing on 2% fructose-YEP medium was used to inoculate 50 ml of a fructose-YEP culture containing 10 μCi of D-[U- 14 C]fructose. Nonradioactive sugars were added to the cultures after growth was established ($A_{550} = 0.3$ to 0.4), and at various times a 0.5-ml sample was mixed with 0.5 ml of 20% TCA, left on ice for 30 min, incubated at 90°C for 30 min, and then filtered through a Whatman microfiber glass filter and washed with 6 ml of 10% TCA and 6 ml of 95% ethanol. Radioactivity on the filters was counted with a Packard Tricarb 2100TR liquid scintillation counter.

Construction and analysis of *glf:lacZ* fusions. A fusion vector, pTC305, was used for construction of in-frame protein fusions of the *Z. mobilis glf* gene to the *Escherichia coli lacZ* gene. An intermediate in the construction, pTC304, was prepared as follows. The multicloning site of pBSSKII+ (Stratagene, Inc.) was modified to delete the *Pst*I site by digestion with *Hinc*II and *Sma*I followed by religation. The modified multicloning site was cut out with *Bss*HIII, the ends were made blunt with Klenow fragment of DNA polymerase, and the site was cloned into the *Sma*I site of the *lacZ* fusion vector pTC261, which was constructed from two other plasmids, pRS414 (23) and pMC1871 (22), as described previously (14). A *Pst*I-to-*Sal*I fragment of pLOI193 (5) was replaced by the *Sal*I-to-*Pst*I fragment from pTC304 to complete construction of pTC305, a broad-host-range shuttle vector that was used for constructing *lacZ* protein fusions. An analogous vector, pTC306, which could be used for making operon fusions, was constructed from pRS415 (23) but was not used in this study. The *glf:lacZ* protein fusion used in this study was constructed as follows. A promoter fragment containing the *Z. mobilis glf* DNA sequence from -228 to +138 with respect to the transcriptional start site (2) and flanked by *Xba*I (upstream) and *Kpn*I (downstream) restriction sites was generated by PCR, as described previously (24). This *glf* promoter fragment was cloned into the *Xba*I-to-*Kpn*I sites of pTC305, resulting in pTC307. The *glf:lacZ* protein fusion plasmid pTC307 was conjugated into *Z. mobilis* CP4 as described previously (5). β -Galactosidase activity was measured essentially as described previously (15), except that cells were broken by passage through a French pressure cell, and activities are reported in micromoles per minute per milligram of total protein. Protein concentrations were measured as described previously (13).

RESULTS

Cometabolism of glucose and fructose. It is believed that *Z. mobilis* preferentially catabolizes glucose for energy and biomass production and preferentially converts fructose to sorbitol and levan as a result of glucose inhibition of fructokinase. Therefore, cometabolism of glucose and fructose during growth under these conditions was tested directly. Incorporation of radioactive fructose into cell biomass (TCA-precipitable cellular components) was used as a measure of catabolism.

FIG. 1. Effects of equimolar glucose on growth and incorporation of radioactive fructose by wild-type *Z. mobilis* CP4. Shown are growth on (top panel) and incorporation of (bottom panel) 2.5% fructose with no additions (circles), with addition of 2.5% nonradioactive fructose (squares), or with addition of 2.5% nonradioactive glucose (triangles). Nonradioactive sugars were added at the time indicated by the arrow. See Materials and Methods for experimental details.

Figure 1 shows the growth and incorporation of radioactive fructose (2.5% or 139 mM) by a culture of the wild-type strain *Z. mobilis* CP4. The addition of an equimolar amount of nonradioactive glucose to the culture resulted in a modest decrease in fructose incorporation compared to a control culture to which an equimolar amount of nonradioactive fructose had been added. The addition of glucose also resulted in a modest stimulation of growth, while the addition of fructose did not. Virtually identical results were obtained with either higher (5%) or lower (0.5%) sugar concentrations (data not shown). These experiments indicated that fructose and glucose were cometabolized, since catabolism and incorporation of fructose continued in the presence of glucose. However, the results did not allow a conclusion as to whether the observed decrease in fructose catabolism from the sugar mixture was the direct result of fructokinase inhibition by glucose.

Since investigation of fructokinase inhibition by glucose in the wild-type strain *Z. mobilis* CP4 is complicated by the presence of glucokinase and glucose catabolism, it was decided to repeat the experiment with a mutant strain, *Z. mobilis* CP4-M2, which is defective in glucokinase and unable to catabolize glucose (6). The addition of nonradioactive glucose to a culture of *Z. mobilis* CP4-M2 growing on radioactive fructose resulted in an immediate and nearly complete cessation of fructose incorporation as well as a substantial decrease in growth rate (Fig. 2). By comparison, the addition of glucose to a culture of the wild type growing on fructose (Fig. 1) had a far less dramatic effect. It should be noted that catabolism by the wild-type strain results in a lower intracellular concentration of

FIG. 2. Effects of equimolar glucose on growth and incorporation of radioactive fructose by the glucokinaseless mutant *Z. mobilis* CP4-M2. Shown are growth on (top panel) and incorporation of (bottom panel) 2.5% fructose with no additions (circles), with addition of 2.5% nonradioactive fructose (squares), or with addition of 2.5% nonradioactive glucose (triangles). Nonradioactive sugars were added at the time indicated by the arrow. See Materials and Methods for experimental details.

FIG. 3. Effects of added glucose on growth and incorporation of radioactive fructose by the glucokinaseless mutant *Z. mobilis* CP4-M2. Shown are growth on (top panel) and incorporation of (bottom panel) 1.0% fructose with no additions (circles) or with addition of 0.125% nonradioactive glucose (triangles). Nonradioactive glucose was added at the time indicated by the arrow. See Materials and Methods for experimental details.

glucose than in the glucokinaseless mutant. In the absence of glucose catabolism in the glucokinaseless strain, the equilibration of glucose by the facilitated transport system (6, 18, 30) would have resulted in an intracellular glucose concentration in *Z. mobilis* CP4-M2 equal to the added concentration (139 mM). In vivo measurements of wild-type *Z. mobilis* (26) indicate that glucose catabolism lowers the intracellular glucose concentration approximately sixfold; it can be assumed that the intracellular glucose concentration in the wild-type *Z. mobilis* CP4 was 23 mM. Thus, the intracellular glucose concentrations in either strain were far in excess of the measured K_i (21) of fructokinase for glucose (0.14 mM), yet fructose catabolism continued in the wild type, indicating that fructokinase remained active (Fig. 1). These results suggested that the effect of glucose on fructose catabolism by the glucokinase mutant involved a mechanism other than (or in addition to) inhibition of fructokinase by glucose.

By adjusting the amount of glucose added to *Z. mobilis* CP4-M2 growing on glucose it was possible to achieve an intracellular glucose concentration lower than that calculated for the wild type in the experiment shown in Fig. 1. As demonstrated in Fig. 3, addition of 0.125% glucose (6.9 mM) to a culture of *Z. mobilis* CP4-M2 growing on 1% fructose (55.5 mM) resulted in a substantial decrease in growth rate and incorporation of radioactive fructose. When *Z. mobilis* CP4-M2 was genetically complemented with a plasmid (pLOI713) containing the cloned *glk* gene (24), the results of glucose inhibition of fructose catabolism were identical to

those of the wild-type strain, confirming that the results shown in Fig. 2 and 3 were attributable to the glucokinase mutation (data not shown). While it might again be argued that the intracellular concentration of glucose (6.9 mM) was in excess of the K_i of fructokinase for glucose, the addition of 0.125% glucose to a culture of *Z. mobilis* CP4 growing on 1% fructose had no effect on growth or incorporation (data not shown). Moreover, an assay of crude extracts of *Z. mobilis* CP4-M2 (Table 1) indicated that fructokinase activity was inhibited only 30 to 36% by a substrate mixture of 10 mM fructose and 1.25 mM glucose, equivalent to a nutrient ratio (1% fructose and 0.125% glucose) which had no effect on growth of the wild type. There was no difference in the total level of fructokinase activity between the two strains (data not shown). In summary, these results suggest that the glucose inhibition of fructose catabolism by *Z. mobilis* CP4-M2 involves a mechanism in addition to that of inhibition of fructokinase.

Effect of nonmetabolizable substrates on fructose catabolism. The only discernible difference between the experiments with *Z. mobilis* CP4 and its glucokinaseless derivative was the nonmetabolizable nature of glucose for the mutant. Therefore, the growth experiments described above were repeated with the nonmetabolizable glucose analogs 3-*O*-methyl glucose and xylose, compounds which do not inhibit fructokinase (7, 21) (data not shown). Addition of an equimolar amount of 3-*O*-methyl glucose, a compound which is known to strongly compete with glucose uptake via Glf (18), to cultures of either *Z. mobilis* CP4 or *Z. mobilis* CP4-M2 growing on fructose resulted in an immediate and nearly complete cessation of growth (Fig.

TABLE 1. Glucose inhibition of fructokinase in crude extracts of *Z. mobilis* CP4-M2

Growth conditions ^a	Fructokinase activity on ^b :					10 mM Glc
	10 mM Fru	10 mM Fru + 1.25 mM Glc	10 mM Fru + 5 mM Glc	10 mM Fru + 10 mM Glc	1.25 mM Fru + 10 mM Glc	
1% Fru	2.18 ± 0.10	1.52 ± 0.02	0.67 ± 0.01	0.36 ± 0.01	0.10 ± 0.005	ND ^c
1% Fru + 0.125% Glc	1.56 ± 0.01	1.00 ± 0.08	0.50 ± 0.05	0.29 ± 0.01	0.06 ± 0.003	ND

^a YEP medium containing the indicated concentrations of sugar(s). Fru, fructose; Glc, glucose.

^b Extracts were assayed with the indicated substrate concentrations as described in Materials and Methods. Fructokinase activity is expressed in micromoles per minute per milligram of total cell protein (mean ± standard deviation).

^c ND, activity not detectable.

4). From this result it was predicted that inhibition of growth by xylose, which is transported via Glf with an apparent affinity equivalent to that of fructose ($K_m = 40$ mM for xylose [25]), would require a significantly higher concentration than glucose ($K_m = 12.5$ mM) or 3-*O*-methyl glucose. In fact, addition of a 50-fold molar excess of xylose to cultures of either *Z. mobilis* CP4 or *Z. mobilis* CP4-M2 growing on fructose resulted in an immediate and nearly complete cessation of growth (data not shown). These results suggest that inhibition of fructose transport was at least partially responsible for the inhibition of fructose catabolism by *Z. mobilis* and that this effect was compounded when the transport analog was nonmetabolizable.

Glf expression and kinetics of glucose and fructose transport. The next step in this study was to directly test the effect of glucose on fructose transport by *Z. mobilis* CP4-M2, but before proceeding it was important to more completely elucidate the transport system(s) involved. It was known that *Z. mobilis* CP4 contains higher levels of *glf*-specific mRNA when

growing on fructose than when growing on glucose (2). In order to determine if the elevated level of *glf* mRNA was the result of transcriptional regulation, a *glf::lacZ* protein fusion containing the *glf* promoter region, ribosome binding site, and first three codons of the *glf* structural gene (2) was constructed. The results shown in Table 2 indicate that the rates of expression of the *glf::lacZ* fusion in mid-logarithmic-phase cultures of *Z. mobilis* CP4 (pTC307) when grown on fructose and when grown on sucrose are 2.5- and 2-fold higher, respectively, than when grown on glucose. Cells grown on a mixture of glucose and fructose showed a level of *glf* expression similar to that obtained on glucose. Stationary-phase cultures generally expressed *glf* at lower levels than cultures in mid-logarithmic phase. The results shown in Fig. 5 indicate that the increased expression of *glf* in fructose-grown cultures of *Z. mobilis* CP4 was accompanied by a 1.5- to 3-fold-higher rate of xylose transport, depending upon the stage of growth (xylose is a proven transport substrate for Glf [6, 30]).

These results clearly demonstrated a connection between *glf* expression and sugar transport, but it remained possible that growth on fructose induced expression of additional fructose transporters. Two previous studies of recombinant *E. coli* strains which overexpressed the *Z. mobilis glf* gene confirmed that Glf transports fructose, but the reports varied significantly in the measured apparent K_m for fructose (>400 mM [18]; 40 mM [30]). It was therefore important to compare the kinetics of sugar transport in *Z. mobilis* CP4 grown on 2% fructose with those on 2% glucose. Glucose-grown cells exhibited an apparent K_m for glucose of 12.5 ± 0.8 mM and a V_{max} of 198 ± 36 nmol/min/mg of protein, and fructose-grown cells exhibited an apparent K_m for glucose of 13.7 ± 2.0 mM and a V_{max} of 405 ± 17 nmol/min/mg of protein. Glucose-grown cells exhibited an apparent K_m for fructose of 224 ± 32 mM and a V_{max} of $1,315 \pm 80$ nmol/min/mg of protein, and fructose-grown cells exhibited an apparent K_m for fructose of 167 ± 41 mM and a V_{max} of $3,000 \pm 284$ nmol/min/mg of protein. The higher V_{max} values for cells grown on fructose are in keeping with the expected increase in *glf* expression, and the lack of significant differences in the K_m values for cells grown on fructose compared to cells grown on glucose strongly suggests that there is

FIG. 4. Effects of added 3-*O*-methyl glucose on growth on fructose by *Z. mobilis* CP4 (top panel) and the glucokinaseless mutant *Z. mobilis* CP4-M2 (bottom panel). Shown are growth on 0.5% fructose with no additions (circles) and that with the addition of 0.5% 3-*O*-methyl glucose (triangles), added at the time indicated by the arrow.

TABLE 2. Expression of a *glf::lacZ* fusion in *Z. mobilis* CP4

Growth phase	β-Gal activity on ^b :			
	10% Fru	10% Glc	5% Fru + 5% Glc	10% Suc
Mid-log ^a	1.65	0.60	0.50	1.50
Stationary	1.20	0.05	0.30	0.45

^a Mid-log phase, $A_{600} = 0.8$ to 1.0 .

^b Cells were grown on YEP medium containing the indicated concentrations of sugar(s). Fru, fructose; Glc, glucose; Suc, sucrose. β-Galactosidase (β-Gal) activity is expressed in micromoles per minute per milligram of total cell protein.

FIG. 5. Increased rate of sugar transport in *Z. mobilis* CP4 grown on fructose. Shown are growth (circles) and transport (squares) of 40 mM xylose by cells grown on 4% fructose (closed symbols) or 4% glucose (open symbols).

only a single transporter in *Z. mobilis* CP4 which is responsible for uptake of both sugars. Eadie-Hofstee plots of fructose transport by fructose-grown cells were linear, further suggesting that there is a single carrier for fructose transport in *Z. mobilis* CP4 (data not shown).

The initial rates of fructose uptake for *Z. mobilis* CP4 and *Z. mobilis* CP4-M2 in the presence and absence of equimolar amounts of glucose were compared (Fig. 6). *Z. mobilis* CP4-M2 took up 30 mM radioactive fructose at a rate of 349 ± 36.6 nmol/min/mg of protein, and the wild type took up 30 mM radioactive fructose at a rate of 499 ± 52.4 nmol/min/mg of protein. Addition of an equimolar amount of nonradioactive glucose resulted in an 84.9% decrease for *Z. mobilis* CP4-M2 (52.8 ± 8.45 nmol/min/mg of protein) and a 74.5% decrease in the rate of fructose uptake by *Z. mobilis* CP4 (127 ± 16.0 nmol/min/mg of protein). Not only was the initial rate of fructose uptake by *Z. mobilis* CP4-M2 exceedingly low, but following equilibration (after 30 s) the rate of fructose uptake dropped to zero (data not shown).

DISCUSSION

Numerous studies have pointed to differential utilization of glucose and fructose by *Z. mobilis* as being physiologically important. The severe inhibition of fructokinase by glucose has been reasoned to result in the accumulation of fructose by *Z. mobilis*, which was made available for levan formation and for conversion to the osmoprotectant sorbitol. The results of fructose incorporation experiments provided by this study clearly indicate that fructose and glucose are cometabolized by wild-type *Z. mobilis* CP4, although the rate of fructose catabolism is lower when glucose is present. While these results do not rule out the involvement of glucose inhibition of fructokinase, it is clear that the *in vivo* inhibition is not as severe as was predicted from *in vitro* enzyme studies. In fact, the data presented in this study indicate that competition for transport plays a very important role in preferential catabolism of glucose from mixtures of fructose and glucose. Moreover, the physiology of fructose metabolism by *Z. mobilis* points to a mechanism that

FIG. 6. Inhibition of fructose uptake by glucose for *Z. mobilis* CP4 (top panel) and *Z. mobilis* CP4-M2 (bottom panel). Shown are levels of uptake of 30 mM radioactive fructose in the presence (open circles) and absence (closed circles) of 30 mM nonradioactive glucose.

allows accumulation of fructose from sucrose and preferential utilization of glucose from sugar mixtures in a way that parallels the need for the osmoprotectant, sorbitol (12), which is formed from fructose.

The unexpected finding that addition of an equimolar concentration of glucose to a culture of the glucokinaseless mutant *Z. mobilis* CP4-M2 resulted in essentially complete inhibition of fructose catabolism, a condition which only slightly affected fructose catabolism by the wild type, led us to believe that mechanisms other than glucose-inhibition of fructokinase were involved in regulating cometabolism of glucose and fructose. The addition of glucose to a level as little as one-eighth of the amount of added fructose still resulted in significant inhibition of growth and incorporation of fructose. The fact that nonmetabolizable transport analogs such as 3-*O*-methyl glucose and xylose, sugars which do not inhibit fructokinase, were able to inhibit fructose catabolism by the wild type led us to hypothesize that competition for sugar transport could prevent catabolism of the excluded sugar. In fact, the results obtained with the nonmetabolizable analogs suggested that competition for transport alone is sufficient to inhibit growth on fructose. In this regard, growth of the wild type on fructose in the presence of strongly competing transport substrates (e.g., 3-*O*-methyl glucose) or high concentrations of poorly competing transport substrates (e.g., xylose) is quite similar to growth of the glucokinaseless mutant in the presence of glucose. The results shown in Fig. 6 demonstrated the profound effect of glucose on the initial rate of fructose transport by *Z. mobilis* CP4-M2.

FIG. 7. Model detailing the roles, locations, and apparent K_m values for various activities involved in glucose and fructose metabolism by *Z. mobilis*. See text for details.

The transport properties of Glf were first characterized in recombinant *E. coli* strains expressing the *Z. mobilis* *glf* gene (18, 30). The present study provides a kinetic analysis of glucose and fructose transport by *Z. mobilis* cells. The V_{max} for fructose transport by *Z. mobilis* was approximately sevenfold higher than the V_{max} for glucose. The apparent K_m for glucose measured in wild-type *Z. mobilis* was approximately 13 mM, and the apparent K_m for fructose was approximately 200 mM, confirming previous measurements by DiMarco and Romano (6) for glucose (K_m , 5 to 15 mM) and by Edye et al. (9) for fructose (K_m , 185 mM). These K_m values, measured in *Z. mobilis*, were approximately fivefold higher than those measured in recombinant *E. coli* strains expressing the *Z. mobilis* *glf* gene (18, 30).

While it has been clear for some time that Glf is a carrier for both glucose and fructose (18, 30), the question of whether or not additional sugar transporters are present in *Z. mobilis* has previously not been fully answered. Cells grown on fructose showed a twofold-higher rate of either glucose or fructose transport (Fig. 5), and there was a corresponding increase in expression of a *glf::lacZ* fusion (Table 2), in addition to higher levels of the *glf* mRNA in cells grown on fructose compared to glucose (2). Kinetic analysis indicated a higher V_{max} for transport of either sugar in cells grown on fructose but, more importantly, no significant differences in the K_m values for either glucose or fructose between *Z. mobilis* grown independently on the two sugars. These results strongly support the conclusion that *Z. mobilis* makes use of a single carrier, Glf, for transport of both glucose and fructose (30). Moreover, all of the effects of glucose, 3-*O*-methyl glucose, and xylose on transport and catabolism of fructose can be explained by competition for transport by a single carrier, Glf.

By definition, the low-affinity, high-velocity facilitated diffusion carrier used by *Z. mobilis* allows rates of sugar influx which are equal to the rates of sugar efflux (6). So long as there is active sugar metabolism, the sugar concentrations inside the cell will be lower than those outside (26) and there is a net influx of sugars. Glf has an affinity for glucose 10-fold higher than that for fructose, and this property alone is sufficient to

explain the preferential uptake of glucose by the wild-type strain. One of the more interesting results of this study was the finding that nonmetabolizable glucose analogs had a profound effect on fructose transport. Recall that fructose transport was severely inhibited by glucose in the glucokinaseless derivative *Z. mobilis* CP4-M2. The effect of nonmetabolizable substrates on transport can be explained by considering the mechanism of facilitated diffusion. Since the rate of glucose efflux is equal to the rate of glucose influx in the absence of glucose catabolism in the glucokinaseless mutant, it can be predicted that both the external and internal sugar binding sites would be primarily occupied by glucose (higher K_m for glucose). In the wild type, where ongoing glucose catabolism results in a lower rate of glucose efflux compared with influx, the external glucose binding site would be occupied by glucose less often. Thus, the competition for fructose influx by glucose would be exaggerated in the absence of glucose catabolism. It should be understood that the profound inhibition of fructose transport by glucose in the glucokinase mutant is not a natural aspect of *Z. mobilis* physiology; rather, it is the consequence of the perturbation of the normal balance of glucose and fructose concentrations in the absence of glucose catabolism.

Model relating transport kinetics to fructose catabolism and osmoregulation. The model shown in Fig. 7 summarizes the kinetics of sugar transport and phosphorylation as well as other relevant aspects of fructose metabolism. In the wild type, the natural difference in affinity for glucose and fructose transport would lead to preferential utilization of the glucose. In addition, glucokinase has a threefold-higher affinity for glucose than fructokinase has for fructose (7, 21), and the latter enzyme is inhibited by glucose (7, 21), though the results of this study indicate that the extent of in vivo inhibition of fructokinase by glucose is not nearly as severe as was predicted from in vitro studies. It follows that the higher the concentrations of glucose and fructose in the growth medium are, the higher the level of extracellular fructose due to preferential glucose catabolism will be. Perhaps even more importantly, given that *Z. mobilis* is most often found in habitats containing high levels of

sucrose (palm sap contains up to 17% sucrose [27]), as glucose and fructose are released from sucrose, *Z. mobilis* would tend to accumulate fructose in the periplasm. The purpose for this fructose accumulation has been made clear by the research of Loos and colleagues (12). The periplasmic enzyme GFOR, which catalyzes the reduction of fructose to sorbitol at the expense of glucose oxidation to gluconate (31), has a very high K_m (>400 mM) for fructose. Growth conditions that result in the accumulation of fructose, in the presence of even significantly lower concentrations of glucose (K_m of GFOR for glucose is 30 mM), would lead to the formation of sorbitol. Sorbitol serves as an osmoprotectant in *Z. mobilis*, is transported with a K_m of 34 mM, and can be accumulated to very high concentrations (12).

A very interesting picture of *Z. mobilis* physiology is developing. This is a simple creature, living in a narrowly defined ecological niche characterized by high sucrose concentrations (27), fermenting only glucose, fructose, and sucrose via the most miserly of energy-yielding schemes (4), and, aside from the modest increase in expression of genes involved in fructose metabolism (2, 10, 32), is apparently devoid of the dramatic changes in global gene expression which allow other bacterial species to adapt to alternative habitats. *Z. mobilis* is an organism which has apparently been optimized during the course of its evolution to do only one thing in order to obtain sufficient energy to support its growth; it rapidly ferments sugars to ethanol and tolerates high concentrations of both. When growing only on fructose, *Z. mobilis* elevates the expression of Glf, fructokinase, and phosphoglucose isomerase in order to support the carbon flux required for rapid growth (1). The high velocity of fructose transport via Glf ensures its rapid catabolism. At high concentrations (transporter-saturated conditions), fructose would be transported ($V_{\max} = 3,000$ nmol/min/mg of protein) more rapidly than it is metabolized (1,000 nmol/min/mg protein [24]) and would equilibrate across the cell membrane, thereby providing osmoprotection. When growing on a mixture of glucose and fructose, *Z. mobilis* expresses Glf, fructokinase, and phosphoglucose isomerase at the lower levels observed during growth on glucose only. Under the former growth condition, cometabolism of glucose and fructose is sufficient for energy generation, and at higher sugar concentrations, GFOR would become saturated, resulting in sorbitol formation for osmoprotection. When growing on sucrose, the expression of Glf, fructokinase, and phosphoglucose isomerase is at a level midway between that observed on glucose alone and that observed on fructose alone. Under conditions of high sucrose concentrations, fructose accumulates to fairly high levels (8, 20), providing for formation of and osmoprotection by sorbitol. This simple, yet elegant system serves *Z. mobilis* well.

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